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TECHNICAL MANUSCRIPT 232

PATHOGENESIS OF THE LETHAL EFFECT OF ANTHRAX TOXIN IN THE RAT:

II. MORPHOLOGIC STUDIES

Frederic G. Dalldorf
Francis A. Beall

JULY 1965

UNITED STATES ARMY
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PATHOGENESIS OF THE LETHAL EFFECT OF ANTHRAX TOXIN IN THE RAT: II. MORPHOLOGIC STUDIES

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Project 1C014501B71A01

July 1965

In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

ABSTRACT

Intravenous injections of anthrax toxin consistently produce pulmonary edema, cyanosis, and death in Fischer 344 rats. A light and electron microscopic study of the lungs of rats sacrificed serially after injections of toxin revealed diffuse pulmonary edema with pronounced capillary endothelial cell changes followed by widespread thrombosis of pulmonary capillaries.

I. INTRODUCTION

It has been known since 1955 that Bacillus anthracis produces a toxin,[1] but its mechanism of action has remained unclear.[2-4] Previous work from this laboratory has shown that Fischer 344 rats are unusually susceptible to intravenous injections of anthrax toxin.[5] The lethal effect of the toxin in these rats is so rapid and predictable that this experimental model has been proposed as a bioassay for anthrax toxins.[6] One of the prominent associated pathologic features in these animals is the development of pulmonary edema. In order to gain additional information concerning the development of this edema, a sequential study of the lung by light and electron microscopy was undertaken.

II. MATERIALS AND METHODS

Male Fischer 344 rats weighing 200 to 300 grams from our colony were used throughout this experiment. The toxin was prepared as previously described[7] and was sterilized by passage through a membrane filter (Millipore®). Each rat received 2 cc of toxin intravenously. This dose was more than that required to kill all rats and regularly resulted in death within 60 to 70 minutes. Most of the rats were sacrificed at 30, 50, and 60 minutes with an intravenous injection of 30 mg of Nembutal®; a few were allowed to die spontaneously of toxin. Some rats from each time period were injected intravenously with 1 cc of a 1:20 dilution of India ink (black drawing ink, S.S. Stafford, Inc., New York, N.Y.) in saline 2 minutes before sacrifice. All injections were made through the dorsal vein of the penis.

Following sacrifice the thorax was opened and the lungs were removed. The right lung was perfused vigorously through the bronchus with Millonig's buffer containing 2% osmium tetroxide. A perfused lobe was sliced with a razor blade and 1-mm cubes of tissue were cut from the central portions of the specimen and placed in additional fixative. Dehydrated blocks were embedded in Epon. Ultrathin sections cut with a diamond knife were stained with uranyl acetate and examined with an RCA EMU-3G electron microscope. One-micron-thick sections were also prepared for light microscopy. The left lung, kidneys, heart, brain, liver, and spleen were removed and placed in 10% buffered formalin. Six-micron-thick paraffin-embedded sections of these tissues were prepared for routine light microscopy.

III. RESULTS

A. THIRTY MINUTES AFTER TOXIN INJECTION

Before being sacrificed 30 minutes after toxin injection, the rats showed no signs of illness. The lungs collapsed upon opening the chest, appeared normal, and were of normal weight. The pleural cavities contained no demonstrable fluid. In the routine sections of the lungs the alveoli were collapsed and the capillaries were filled with erythrocytes and contained no carbon particles (Fig. 1). The adventitia around the pulmonary arteries and veins was collapsed and narrow. Routine histologic sections of all other organs were essentially normal. The one-micron sections of the lungs also appeared normal (Fig. 2). The pulmonary capillaries were filled with blood and the endothelial cells lining the capillaries, the alveolar epithelial cells, and the alveolar macrophages were clearly discernible.

Electron microscopic examination of the lungs revealed normal pulmonary ultrastructure.[8] The capillary walls were completely lined by a thin cytoplasmic extension of the endothelial cells and the alveoli were lined by a continuous layer of epithelial cell cytoplasm. The basement membrane of the pulmonary capillaries appeared as a thin, homogeneous, electron-dense structure separating these two cytoplasmic layers. Both epithelial and endothelial cells contained many small, pinocytotic vesicles as well as the usual cytoplasmic organelles. When the cytoplasmic processes of adjacent epithelial or endothelial cells met, the cell walls were thickened to form desmosomes (Fig. 3).

B. FIFTY MINUTES AFTER TOXIN INJECTION

Fifty minutes after toxin injection the rats showed early signs of the lethal effect of the toxin, consisting of the gradual development of rapid shallow respiration. After sacrifice the lungs were voluminous and heavy. There were small hyperemic areas measuring up to 2 mm scattered throughout the pulmonary parenchyma. The pleural cavities contained little or no fluid. There was no gross evidence of carbon staining of the lungs in the rats injected with ink. The microscopic examination of routine sections of the lungs showed evidence of widespread pulmonary edema (Fig. 4). Many of the alveoli were filled with fluid and there was marked perivascular edema. An occasional rare capillary filled with carbon particles was seen in the sections of lung injected with ink. Light microscopic study of the one-micron-thick sections of the lung revealed some subtle changes in the pulmonary capillaries. The lumen of many capillaries contained very few erythrocytes and were often bridged by delicate, pale staining membranes (Fig. 5). Electron micrographs revealed these membranes to be reflections of the cytoplasmic processes of the capillary endothelial cells (Fig. 6 and 7). Large clear spaces had formed between the thin cytoplasmic

processes of many endothelial cells and the underlying basement membrane. These spaces occasionally contained some cytoplasmic fragments but were usually filled with structureless material. Carbon particles were seen neither within these subendothelial spaces nor along the exposed basement membranes. The rare carbon-filled capillaries seen in the routine light microscopic sections of the lung were not found in the much smaller sections prepared for electron microscopy. No changes were observed in the basement membranes, the junctions between adjacent endothelial cells, the alveolar epithelial cells, or the endothelial cells lining the pulmonary veins and arteries.

C. SIXTY TO SEVENTY MINUTES AFTER TOXIN INJECTION

Sixty to seventy minutes after toxin injection the rats were near the terminal stage of their illness. They assumed a prone position and had rapid labored respirations. By this time all rats had cyanosis of the feet and eyes. At death frothy fluid often poured out of the nose. The lungs were very heavy and contained many hemorrhagic areas measuring up to 3 mm. There were many small areas of carbon staining in the lungs of the rats injected with ink. Each pleural cavity contained about 1 cc of clear fluid. The routine histologic sections of the lungs showed evidence of marked alveolar and perivascular edema and some hemorrhage. Most of the pulmonary capillaries and some of the small veins were filled with clumps of carbon particles (Fig. 8). The number of capillaries filled with carbon particles increased as the rats neared the terminal stage of illness. The larger pulmonary blood vessels contained no carbon. The only morphologic changes observed in the other organs consisted of occasional carbon-filled capillaries in the renal glomeruli, the heart, and the brain.

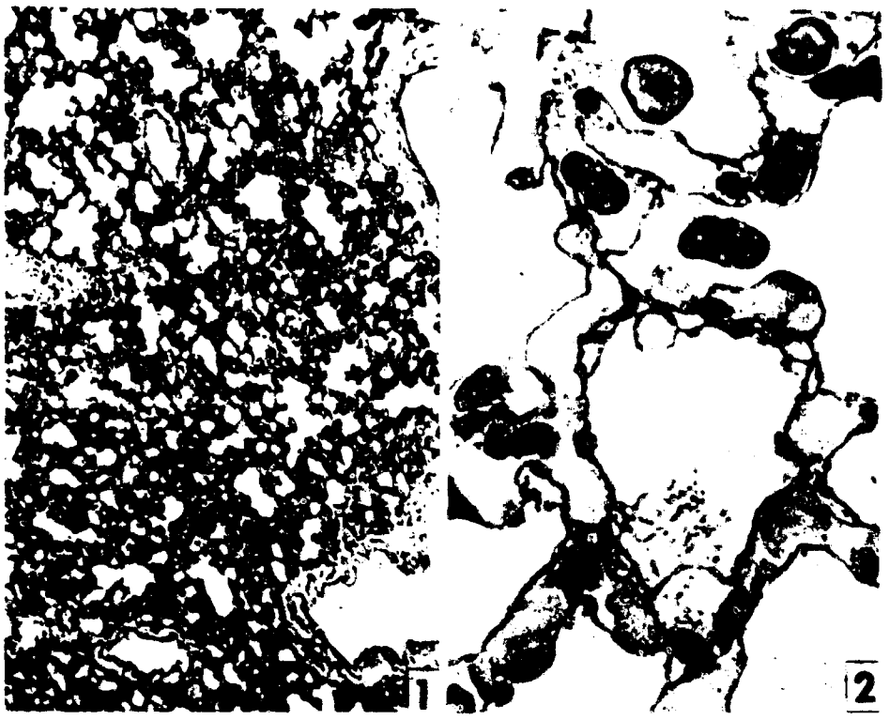
Light microscopic examination of the one-micron-thick sections of the lung showed that the ink particles were trapped in small granular thrombi within the lumen of many capillaries (Fig. 9). Electron micrographs revealed these thrombi to be composed largely of platelets[8] (Fig. 10) and granular fibrin containing carbon particles (Fig. 11). The cytoplasmic processes of the endothelial cells were intact in these thrombosed capillaries. Numerous large subendothelial spaces still remained a prominent feature in the terminal stages of the disease (Fig. 11). Occasional capillaries contained ink-laden macrophages. There was no definite evidence of endothelial cell lysis and no morphologic changes were observed in the endothelial cell junctions, the basement membranes, the alveolar epithelial cells, or the endothelial cells lining the pulmonary arteries and veins. Numerous thrombi were also seen in the pulmonary capillaries of terminal rats not injected with ink.

These first three figures illustrate material taken from the lung of a rat sacrificed 30 minutes after intravenous injection of 2 cc of toxin and 2 minutes after the intravenous injection of India ink.

Figure 1. The Lung Appears Normal in this Routine Unperfused Section. Many alveoli are collapsed and there is no evidence of perivascular edema or carbon staining of the pulmonary capillaries. Hematoxylin and eosin. X 70.

Figure 2. One-Micron-Thick Section Showing Normal Pulmonary Capillaries. Hematoxylin. X 1,200.

Figure 3. Electron Micrograph of the Wall of a Pulmonary Capillary. The capillary lumen is almost filled with an erythrocyte (Rbc). The endothelial (End) and epithelial (Epi) cell cytoplasmic processes are intact. A normal junction (Jct) between adjacent endothelial cells is thickened to form a desmosome. The capillary basement membrane appears as a thin electron-dense layer separating the two cytoplasmic processes. Uranyl acetate. X 27,000.



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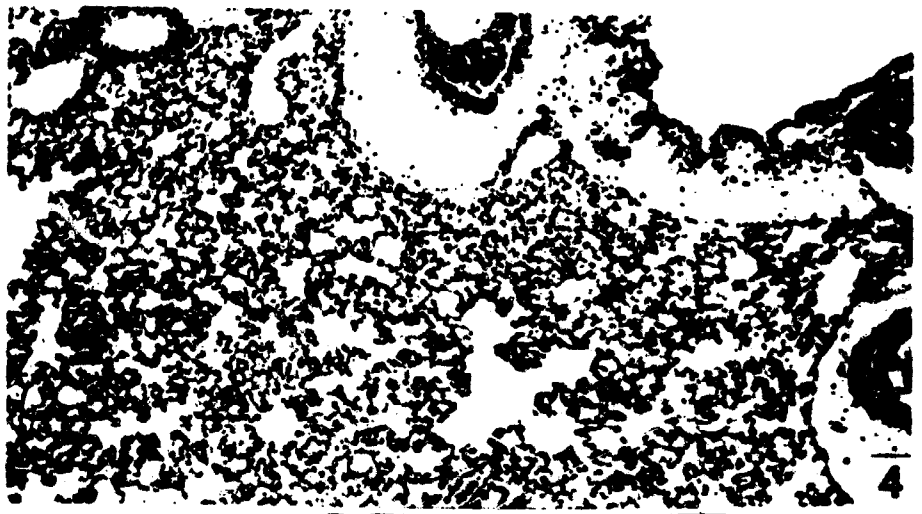
These three photomicrographs were taken from the lung of a rat sacrificed 50 minutes after the intravenous injection of 2 cc of toxin and 2 minutes after the intravenous injection of India ink.

Figure 4. Routine Histologic Section of the Unperfused Lung. The alveoli are partly filled with fluid and there is perivascular edema. There are no carbon-filled capillaries in this photograph. Hematoxylin and eosin. X 70.

Figure 5. The Capillaries in this One-Micron-Thick Section Contain very Few Erythrocytes and Their Lumen are Often Bridged by Thin Pale Membranes. Basic fuchsin. X 1,100.

Figure 6. The Capillary Lumen (Cap) Contains Two Large Vesicular Structures Resulting from the Accumulation of Fluid Between the Thin Cytoplasmic Processes of the Endothelial Cell and the Basement Membrane. The endothelial cell junction (Jct) is intact. Uranyl acetate. X 12,700.



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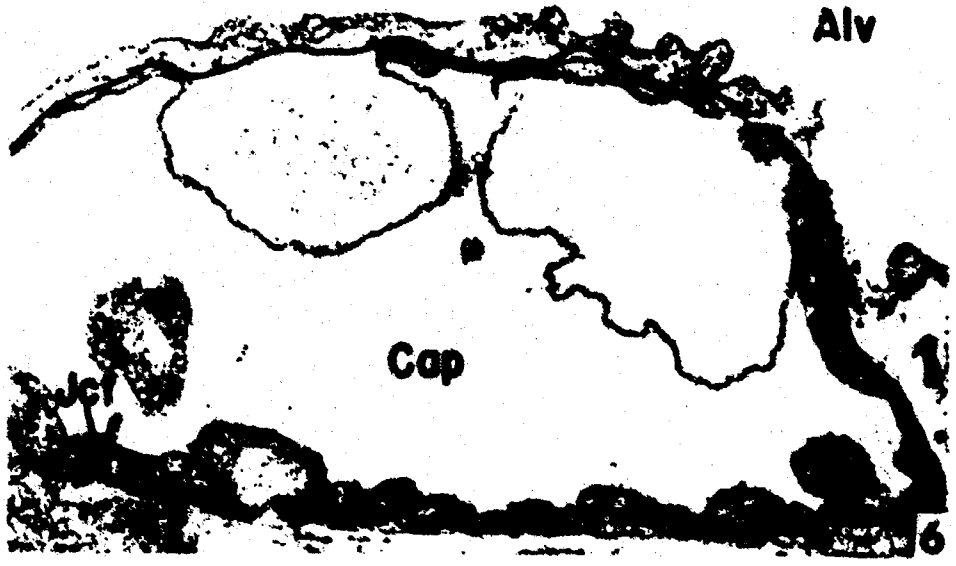

Cap

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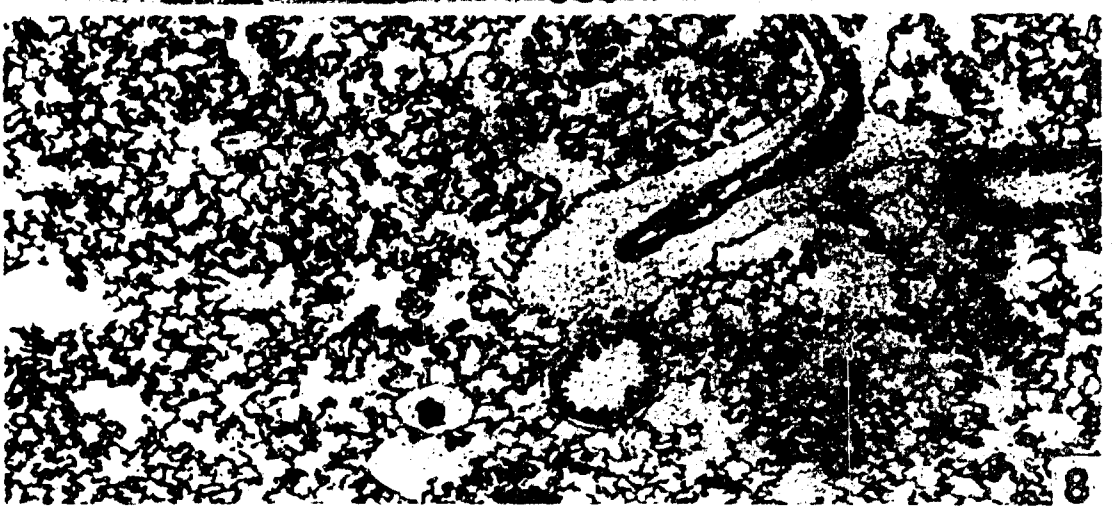
Figure 7. The Cytoplasmic Process of this Endothelial Cell (End) has Separated from Its Basement Membrane and is Deflected Across the Capillary Lumen (L), thus Forming a Large Subendothelial Space (S). The endothelial cell has no other changes indicative of cytolysis. The epithelial cell membrane (Epi) is normal and the epithelial cell junction (Jct) is unaltered. Uranyl acetate. X 19,000.

Figures 8 and 9 are Photographs of Sections Taken from Lungs of Rats 60 to 70 Minutes after Receiving Toxin and 2 Minutes after Being Injected with India Ink.

Figure 8. This Section is from the Lung of a Rat that Died 70 Minutes after Receiving Toxin. There is marked perivascular and alveolar edema. Most of the pulmonary capillaries are filled with black ink particles. Hematoxylin and eosin. X 70.

Figure 9. This One-Micron-Thick Section has Many Pulmonary Capillaries Filled with Small Granular Thrombi that Contain Ink Particles. Basic fuchsin. X 1800.

7



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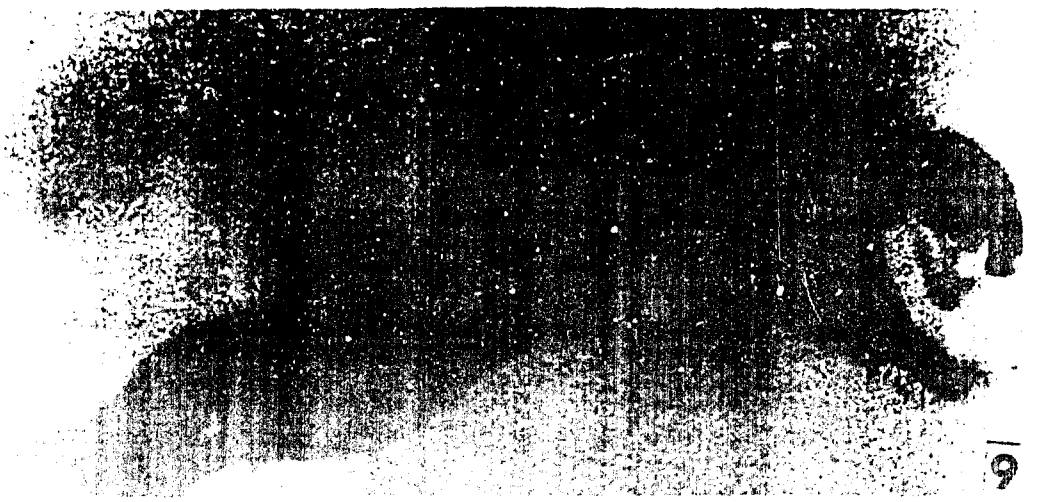

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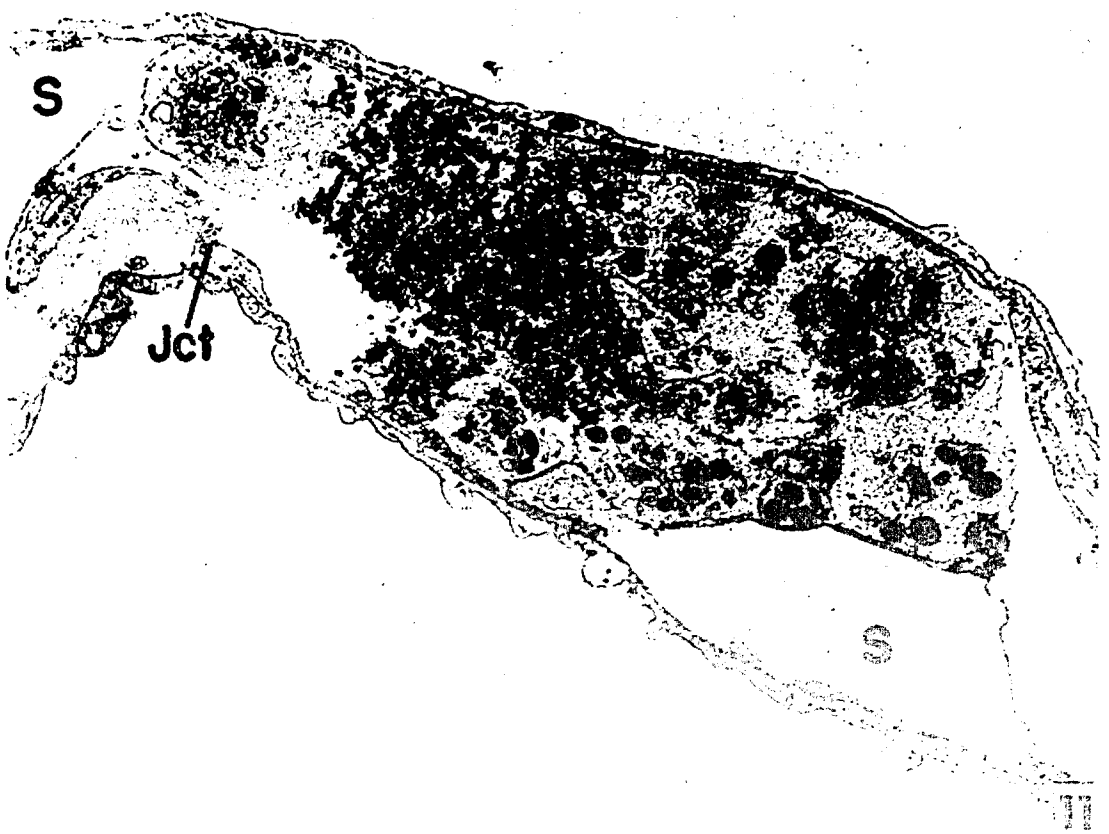
Figures 10 and 11 show photomicrographs of sections taken from lungs of rats 60 to 70 minutes after receiving toxin and 2 minutes after being injected with India ink.

Figure 10. This Pulmonary Capillary (Cap) is Occluded by a Platelet Thrombus Containing Clumps of Electron-Dense Carbon Particles (C). The endothelial cell cytoplasm is intact. Uranyl acetate. X 10,000.

Figure 11. The Lumen of this Pulmonary Capillary Contains a Large Macrophage (M) with Many Dark Carbon Particles. A platelet thrombus partly occludes the lumen. The endothelial cell membrane is separated from the basement membrane in one area. The endothelial cell junction (Jct) is intact. Uranyl acetate. X 13,000.


10


S
Jct
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IV. DISCUSSION

Recent studies have shown that most examples of increased vascular permeability can be divided into two categories.[9] The first consists of those experimental models in which vascular leakage is induced by endogenous mediators such as histamine or serotonin and are characterized by their immediate onset and transient response. Electron microscopic studies of these experimental models have been unable to demonstrate any alterations in capillary endothelium but have shown a separation of the endothelial cells lining the venules. The second type of vascular leakage results from the direct effect of various agents upon the capillary endothelial cells. Examples of this type of increased permeability are those caused by bacterial toxins,[10] aminonucleoside,[11] and heat.[9] The vascular leakage in these experimental models is characterized by a delayed onset and a sustained response. Electron microscopic studies of these experimental models have demonstrated alterations in the endothelial cells lining the capillaries.[11-18]

The present experimental model would best belong to the latter group of reactions. Physiologic studies of the pulmonary, peritoneal, and subcutaneous vascular beds have shown that the vascular leakage caused by anthrax toxin has a delayed onset and a sustained response.[7] The electron microscopic studies of the lungs show a marked alteration of capillary structure, which coincides with the rapid development of pulmonary edema.

The experimental evidence presented here indicates that anthrax toxin causes the rapid death of Fischer rats by altering the physical properties of the membranes of the endothelial cells lining the pulmonary capillaries, which results in increased permeability with diffuse pulmonary edema and widespread capillary thrombosis. The rats apparently die of hypoxia caused by pulmonary edema and pulmonary capillary thrombosis.

LITERATURE CITED

1. Smith, H.; Keppie, J.; Stanley, J.L. 1955. The chemical basis of the virulence of Bacillus anthracis: V. The specific toxin produced by B. anthracis in vivo. Brit. J. Exp. Pathol. 36:460-472.

2. Eckert, N.J.; Bonventre, P.F. 1963. In vivo effects of Bacillus anthracis culture filtrates. J. Infect. Dis. 112:226-232.

3. Bonventre, P.F.; Eckert, N.J. 1963. The biologic activities of Bacillus anthracis and Bacillus cereus culture filtrates. Amer. J. Pathol. 43:201-211.

4. Nordberg, B.K.; Schmiterlow, C.G.; Hansen, H.J. 1961. Pathophysiological investigations into the terminal course of experimental anthrax in the rabbit. Acta Pathol. Microbiol. Scand. 53, Fasc. 3, 295-318.

5. Beall, F.A.; Taylor, M.J.; Thorne, C.G. 1962. Rapid lethal effect in rats of a third component found upon fractionating the toxin of Bacillus anthracis. J. Bacteriol. 83:1274-1280.

6. Haines, B.W.; Klein, F.; Lincoln, R.E. 1965. Quantitative assay for crude anthrax toxins. J. Bacteriol. 89:74-83.

7. Beall, F.A.; Dalldorf, F.G. 1965. Pathogenesis of the lethal effect of anthrax toxin in the rat: I. Physiologic and pharmacologic studies, (Technical Manuscript 231). Pathology Division, U.S. Army Biological Laboratories, Frederick, Maryland.

8. Schulz, H. 1959. Die Submikroskopische Anatomie und Pathologie der Lung. Springeer-Verlag, Berlin. 199 p.

9. Cotran, R.S.; Majno, G. 1964. The delayed and prolonged vascular leakage in inflammation: I. Topography of the leaking vessels after thermal injury. Amer. J. Pathol. 45:261-281.

10. Elder, J.M.; Miles, A.A. 1957. The action of lethal toxins of gas-gangrene clostridia on capillary permeability. J. Pathol. Bacteriol. 74:133-145.

11. Friederici, H.H.R.; Pirani, C.L. 1964. The fine structure of peripheral capillaries in experimental nephrotic edema. Lab. Invest. 13. 250-258.

12. Cotran, R.S.; Majno, G. 1964. A light and electron microscopic analysis of vascular injury. Ann. N.Y. Acad. Sci. 116:750-764.

13. Cotran, R.S. 1965. The delayed and prolonged vascular leakage in inflammation: II. An electron microscopic study of the vascular response after thermal injury. Amer. J. Pathol. 46:589-620.

Unclassified

Security Classification

DOCUMENT CONTROL DATA - R&D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1 ORIGINATING ACTIVITY (Corporate author)

U.S. Army Biological Laboratories
Fort Detrick, Frederick, Maryland, 21701

2a REPORT SECURITY CLASSIFICATION

Unclassified

2b GROUP

3 REPORT TITLE

PATHOGENESIS OF THE LETHAL EFFECT OF ANTHRAX TOXIN IN THE RAT:
II. MORPHOLOGIC STUDIES

4 DESCRIPTIVE NOTES (Type of report and inclusive dates)

5 AUTHOR(S) (Last name, first name, initial)

Dalldorf, Frederic G.
Beall, Francis A.

6 REPORT DATE	7a TOTAL NO. OF PAGES	7b NO. OF REFS
July 1965	20	13

8a CONTRACT OR GRANT NO.	9a ORIGINATOR'S REPORT NUMBER(S)
b PROJECT NO.	Technical Manuscript 232
c 1C014501B71A01	9b OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d	

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11 SUPPLEMENTARY NOTES	12 SPONSORING MILITARY ACTIVITY
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